IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Yunping Li
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Examiner: Phyllis G. Spivack
Group Art Unit: 1641
Docket No.: BBRI-2008US01

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with The United States Postal Service as First Class Mail in an envelope Addressed to Commissioner of Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 5/19/06

5/19/06

DECLARATION UNDER 37 CFR 1.132

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I Kathleen Morgan declare and state as follows:

1. I Kathleen Morgan and Yunping Li are the inventors of the subject matter claimed in U.S. Application Number 10/620,317.

2. Prior to making this Declaration I studied the following documents:
   a) U.S. Application Number 10/620,317;

b) the Office Action mailed from the Patent Office on January 19, 2006 in connection with U.S. Application Number 10/620,317; and

c) Li, *et al.*, Anesthesiology, (April 2003) Vol. 98, Supp. 1, pp 11, as cited in the Office Action.

3. The rejections of Claims 1-11 specified in the above-referenced Office Action have been explained by patent counsel, including the provisions of 35 USC § 102(a), which relates to the novelty requirement. The specified grounds for rejection of the Claims were also discussed.

4. It is my understanding that the rejection stated in the outstanding Office Action is based on the Examiner's impression that the invention as claimed was conceived by an inventive entity other than those named in the instant application. This rejection is based on the belief that all of the named persons in the reference cited above had conceived aspects of the invention as claimed. This is not the case – only those named as inventors in the instant application had conceptual input into the invention as claimed. One author of the cited abstract not named as an inventor in the subject patent application is Sabah Malek. She was a summer student working in a technical capacity under my direction and made no conceptual contribution to the invention. The other author not named as an inventor on the subject patent application is Hyun-Dong Je. He was an post doctoral fellow conducting routine assays under my direction and made no conceptual contribution to the invention.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

| | |
|---|---|
| Signature | Kathleen Morgan |
| Name | Kathleen Morgan |
| Date | 5-17-06 |

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chapter 22

# Pharmacological inhibitors of the ERK signaling pathway: application as anticancer drugs

**Michiaki Kohno[1] and Jacques Pouyssegur[2]**

[1] Laboratory of Cell Regulation, Department of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-14, Bunkyo-machi, Nagasaki 852-8521, Japan.
[2] Institute of Signaling, Developmental Biology and Cancer Research, CNRS-UMR 6543, Centre A. Lacassagne, 33 Avenue Valombrose, 06189 Nice, France.
* To whom correspondence should be addressed

**The ERK signaling pathway, also known as the p42/p44 MAP kinase pathway, is a major determinant in the control of cell growth, cell differentiation and cell survival. This pathway, which operates downstream of Ras, is often up-regulated in human tumors and as such represents an attractive target for anticancer therapy. In this chapter we review the rationale for targeting the components of the ERK pathway, either alone or in association with cytotoxic anticancer agents. We present the most advanced inhibitors of this pathway and discuss their specificity and mechanism of action.**

## INTRODUCTION

Receptor tyrosine kinases, cytokine receptors and some G protein-coupled receptors activate intracellular protein serine/threonine kinases termed mitogen-activated protein kinases (MAPKs). The activation of MAPKs requires a cascade-like mechanism in which each MAPK is phosphorylated by an upstream protein kinase, MAPK kinase (MAPKK), and the latter in turn is phosphorylated by a third protein kinase, MAPK kinase kinase (MAPKKK). There are at least three such protein kinase modules in mammalian cells. These are the Extracellular signal-Regulated Kinase (ERK) module, also known as the p42/p44 MAPK module, the c-Jun amino-terminal kinase (JNK) module, and the p38 MAPK module (1-3).

The ERK pathway is the most thoroughly studied of the cytoplasmic signaling pathways. Activation of this pathway involves the GTP-loading of Ras at the plasma membrane, and the sequential activation of a series of protein kinases (Figure. 1). Initially, activated Ras recruits the Raf family of kinases such as Raf-1 to the plasma membrane, a key step in a complex activation process not yet fully resolved. Raf-1 acts as a MAPKKK and activates MAP kinase/ERK kinase 1 and 2 (MEK1/2; also called MKK1/2) by serine phosphorylation. MEK1/2, dual-specificity protein kinases, then catalyze the phosphorylation of ERK1 and ERK2 (p44 MAP kinase and p42 MAP kinase, respectively) on tyrosine and threonine residues. When activated, ERK1/2 phosphorylate various downstream substrates involved in a multitude of cellular responses from cytoskeletal changes to gene transcription. Although the ERK pathway, like the JNK pathway and the p38 MAPK pathway, participates in the regulation of a wide range of biological processes, it has been linked particularly to the control of cell proliferation, cell differentiation and cell survival (1-3).

Very precise spatio-temporal control mechanisms for MAPK activities have evolved to ensure homeostasis in multicellular organisms. Accordingly, inappropriate activation of these MAPK pathways might result in the induction of several diseases. Aberrant activation of the ERK pathway, for example, has been shown to be an essential feature common to many types of human tumors (4-7). Thus, there has been an explosion of interest in the components of MAPK signaling pathways as attractive therapeutic targets for specific applications (8, 9).

In this chapter, the current status of inhibitors of the ERK pathway will be presented, with focus on the potential application of small-molecule inhibitors of Raf-1 and MEK1/2 as anticancer drugs. Pharmacological inhibitors have also been developed that affect the JNK and p38 MAPK pathway. These inhibitors have been examined for possible application as anti-inflammatory drugs but not as anticancer drugs. We refer the reader to other reviews for a broader overview of this subject (8).

## RATIONALE FOR TARGETING THE COMPONENTS OF THE ERK PATHWAY AS ANTICANCER DRUGS

The ERK pathway is activated by a wide variety of mitogenic stimuli which interact with structurally distinct receptors and thus represents a convergence point for most, if not all, mitogenic signaling pathways (1-3). We have established that activation of ERK1/2 is crucial for cyclin D1 induction, providing a molecular link between ERK signaling and cell cycle control (10). Therefore, it is not surprising that specific blockade of the ERK pathway abolishes cell growth. This was demonstrated by two independent approaches, one by transient expression of an ERK-specific antisense (11), the second by sequestering ERK1/2 in the cytoplasm and therefore preventing ERK nuclear signaling (12).

**Specific inhibition of the ERK pathway is expected to commonly and quite effectively intercept a variety of mitogenic signals.**

Aberrant activation of signal-transducing proteins has been linked to cancer. For instance, overexpression

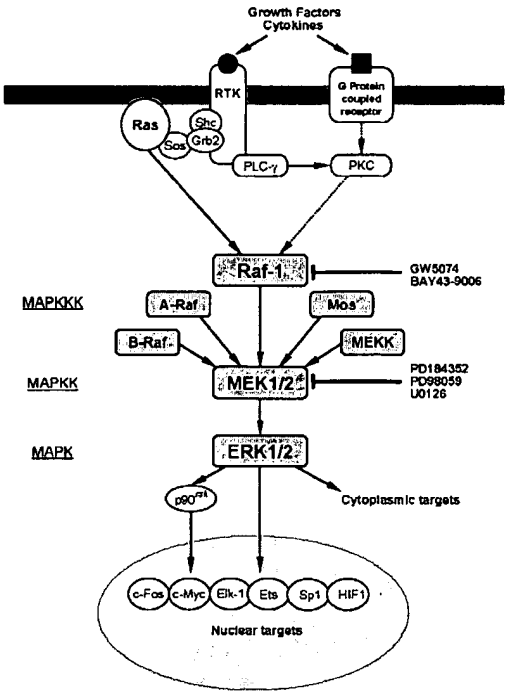


**Figure 1. Schematic representation of the ERK pathway focusing on its function at the convergence of diverse intracellular signaling pathways.**
The MAP kinase cascade contains three sequential kinases: MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK). Activation of the ERK pathway is most often associated with cell proliferation and cell survival. Well-characterized inhibitors of Raf-1 and MEK1/2 are also shown.

of the EGF receptor (c-*erb* B-1) gene or constitutively active mutants of Ras have been observed in several human tumors, and constitutively active mutants of MEK1 have been shown to transform mammalian cells (13). Furthermore, constitutive activation of ERK1/2 and MEK1/2 has been detected in a relatively large number of tumors; tumor cells derived from pancreas, colon, lung, ovary and kidney show especially high frequencies (30~50%) of kinase activation. Although the precise cause of constitutive activation of the ERK pathway in the majority of such tumor cells remains unclear, it seems very likely not to result directly from a disorder of ERK1/2 or MEK1/2, but rather from a disorder in Raf or other upstream signaling molecules (4). As the ERK pathway represents a convergence point for the majority of mitogenic signaling pathways, there are many upstream signaling molecules, such as receptor tyrosine kinases (RTKs) Grb2, Sos, Shc, Ras and protein kinase C, whose abnormal activation could culminate in the constitutive activation of the ERK pathway and which could represent potential targets for the development of anticancer drugs. Accordingly, specific inhibitors of the EGF receptor tyrosine kinase (14) and Ras (15), for example, are currently being developed and examined for therapeutic application as anticancer drugs.

Mutated gene products with elevated activity themselves do not necessarily represent the most suitable therapeutic targets. Thus, although mutation of genes encoding for MEK1/2 or ERK1/2 has rarely been detected in human tumors, these signaling molecules also represent excellent targets for the development of anticancer drugs. Because of their converging function (Figure 1), specific inhibition of MEK1/2 or ERK1/2 is expected to quite effectively intercept a wide variety of upstream aberrant mitogenic signals. The MEK inhibitor PD98059, for example, totally inhibits the proliferation of HT1080 fibrosarcoma cells in which the ERK pathway is constitutively activated as the result of aberrant activation of Ras (16).

**Inhibition of the ERK pathway is expected to result in anti-metastatic as well as anti-angiogenic effects.**

Specific blockade of the ERK pathway in colon tumor cells has been shown to inhibit the alterations in cell-cell contact and motility that are required for metastasis (17). This finding is consistent with the observation that the ERK pathway plays an essential role in the induction of epithelial cell motility in response to hepatocyte growth factor (HGF) (18). HGF-induced activation of the ERK pathway has been linked to the expression of the *matrix metalloproteinase* (*mmp*)-*9* gene, and MMP-9 activity is required for the induction of cell motility (19). In this respect, elevated expression of MMPs has been associated with increased metastatic potential in many tumor cells, and inhibition of MMP activity results in reduction of tumor invasion and metastasis (20, 21). Furthermore, transfection of a constitutively active form of MEK1 has induced increased expression of MMP2/9 and confers metastatic potential to NIH3T3 cells (22).

Vascular endothelial growth factor (VEGF), a potent angiogenic factor, is overexpressed in a variety of tumor cells and contributes to their neo-vascularization (23). The ERK pathway plays a critical role in the transcriptional regulation of VEGF (24). In addition, sustained activation of the ERK pathway has been reported to be required for basic fibroblast growth factor-induced angiogenesis (25). Furthermore, activation of the ERK pathway occurs in response to integrin-mediated cell adhesion to the extracellular matrix, which plays a critical role in both tumor metastasis and angiogenesis (26).

All these observations thus indicate that, in addition to anti-proliferative effects, specific inhibition of the ERK pathway is expected to result in anti-metastatic and anti-angiogenic effects in tumor cells.

**Inhibition of the ERK pathway enhances the anticancer effect of cytotoxic drugs.**

MEK inhibitors have been shown to enhance the lethal action of diverse cytotoxic anticancer agents such as ara-C (a deoxycytidine analogue which inhibits DNA replication), cisplatin (a DNA-reactive agent which induces intrastrand crosslinks), paclitaxel/taxo-

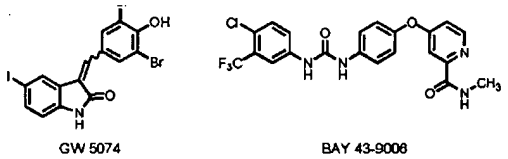


**Figure 2. Structures of published Raf-1 inhibitors.**

tere (taxanes which stabilize microtubules) and vinblastine/vincristine (*vinca* alkaloids which bind to tubulin subunits and inhibit tubulin polymerization) (27). All these agents induce the activation of the ERK pathway as well as JNK/p38 MAPK pathways. It has generally been suggested that activation of the ERK pathway is associated with anti-apoptotic processes, while activation of the JNK/p38 MAPK pathways is linked with pro-apoptotic processes. It has therefore been proposed that the balance between the ERK pathway and JNK/p38 MAPK pathways determines the fate of cells after various stresses (28).

The ERK pathway has been linked to, for example, the phosphorylation of Bcl-2 that contributes to cell survival (29), the suppression of the apoptotic effect of BAD (30), the accumulation of the p53 tumor suppressor protein (31), and the up-regulation of the anti-apoptotic protein MCL-1 (32). Thus, although the precise mechanisms by which a combination of MEK inhibitors and cytotoxic drugs induces enhanced anticancer effects are largely unknown, specific interruption of the putatively cytoprotective ERK pathway could, by shifting the balance between pro- and anti-apoptotic signaling, enhance the lethal actions of established cytotoxic anticancer drugs.

## SPECIFIC INHIBITORS OF THE ERK PATHWAY

### Raf-1 inhibitors.

The major cause of constitutive activation of the ERK pathway in human tumors is a disorder in Raf, Ras or other signaling molecules upstream of Ras (4). For example, a recent report has shown that activating mutations of B-Raf are detected in several human cancer cell lines, including melanomas (59%), colorectal carcinomas (18%), gliomas (11%) (33). Specific inhibitors of Raf are expected to efficiently block such aberrantly activated mitogenic signaling. Potent inhibitors of Raf-1 have recently been developed; they are GW5074 and BAY 43-9006 (Figure 2).

GW5074 was identified as a Raf-1 inhibitor by screening more than 200 compounds in the benzylidene oxindole series using a cascade assay of three non-homologous kinases, Raf/MEK/ERK2, (34). GW5074 inhibits Raf-1 kinase activity *in vitro* with an $IC_{50}$ of 9 nM. It also inhibits EGF-stimulated ERK activation quite effectively without inhibiting the EGF receptor tyrosine kinase.

BAY 43-9006 was identified as a potent inhibitor of Raf-1 by screening many thousands of chemical compounds using a combination of an *in vitro* Raf kinase biochemical assay and a tumor cell-based mechanistic assay (35). BAY 43-9006 inhibits Raf-1 kinase activity *in vitro* with an $IC_{50}$ of 12 nM. It also suppresses tumor growth in human tumor xenograft models with mutant K-*ras* genes (HTC116 colon carcinoma, MiaPaca-2 pancreatic carcinoma and H460 non-small cell lung carcinoma) and with a wild-type K-*ras* but exhibiting overexpression of HER 2 (SKOV-3 ovarian carcinoma). BAY 43-9006 is the first orally active compound in this class and is currently in Phase I clinical trials in locally advanced or metastatic cancers.

In another approach, ISIS 5132, a 20-base phosphorothioate antisense oligodeoxynucleotide designed to hybridize to the 3' untranslated region of the c-*raf*-1 mRNA, has been successfully employed as a possible anticancer agent (36). ISIS 5132 displayed an $IC_{50}$ between 50 and 100 nM in inhibiting *c-Raf-1* expression and tumor cell proliferation in culture, and an $IC_{50}$ between 0.06 and 0.6 mg/kg in inhibiting tumor growth *in vivo* when administrated once daily by intravenous injection. The cell lines examined were A549 lung carcinoma cells, MDA-MB-231 beast carcinoma cells and T24 bladder carcinoma cells. Early clinical data have shown that ISIS 5132 is well tolerated and specifically suppresses c-Raf-1 expression in patients with advanced cancers (37, 38).

### MEK1/2 inhibitors.

Although Raf-1 is the major activator of MEK1/2, these kinases are also activated by several other kinases such as Mos, A-Raf, and B-Raf. On the other hand, no substrates for MEK1/2 have been identified other than ERK1 and ERK2. This tight selectivity, in addition to an ability to phosphorylate both tyrosine and threonine residues, is consistent with MEK1/2 playing a central role in the integration of mitogenic signals into the ERK pathway (1-3). While MEK1/2 have not been identified as oncogene products, they stand at the focal point of many mitogenic signaling pathways (Figure 1), and constitutive activation of MEK1 has been detected in a variety of human tumor cells (4). All these finding highlight MEK1/2 as excellent

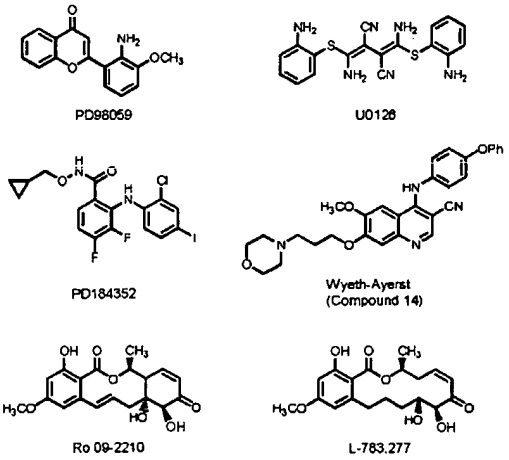


**Figure 3. Structures of published MEK1/2 inhibitors.**

targets for the development of therapeutic agents against cancer (8, 9). Specific and potent MEK1/2 inhibitors have been developed (Figure 3).

PD98059, the first MEK inhibitor, was identified by screening a compound library with an *in vitro* cascade assay that measured the inhibition of ERK1 activation by a constitutively active mutant MEK1 recombinant protein (39). Meanwhile, U0126 was discovered by screening a total of 40,000 compounds in a cell-based reporter assay that measured the inhibition of phorbol ester-stimulated AP-1 transactivation (40). These first-generation MEK inhibitors have been utilized extensively to elucidate the role of the ERK pathway in a variety of biological processes. A second-generation MEK inhibitor with enhanced bioavailability, PD184352, has been synthesized (17 ).

The majority of protein kinase inhibitors developed so far are competitive with ATP and are believed to interact within the ATP-binding site of their target protein kinase. PD98059, U0126 and PD184352 differ in this respect since they do not compete with ATP. Such a peculiar characteristic may allow PD98059, U0126 and PD184352 to work as extremely specific inhibitors of MEK1/2; none of these compounds significantly inhibits the activity of a large panel of protein kinases which includes ERK1, JNK1 and p38 MAP kinases in an *in vitro* assay (41).

Their precise action mechanism of PD98059, U0126 and PD184352 as MEK inhibitors remains controversial. Some confusion was introduced by the different experimental conditions employed in each study, *i.e.*, use of purified recombinant enzymes versus immunoprecipitated enzymes or wild-type enzymes (activated) versus constitutively-active mutant enzymes. In fact, initial reports suggested that PD98059 does not inhibit MEK1 activity directly but inhibits its activation (phosphorylation) by Raf-1 (42), while U0126 was reported to inhibit MEK1/2 activity directly, equilibrium binding studies indicate that PD98059 and U0126 bind MEK1 at the same or overlapping sites (40), but not the phosphorylation of MEK1 by Raf-1. However, equilibrium binding studies indicate that PD98059 and U0126 bind MEK1 at the same or overlapping sites (40). Accordingly, recent studies have shown that U0126 and PD98059 act similarly: both prevent phosphorylation of MEK1 by upstream kinases in a manner that appears to be substrate-directed (41, 43).

PD184352 inhibits activation of MEK1 in cells by 50% at 2 nM, a concentration over 100-fold lower than that which inhibits MEK1 activity *in vitro* (41). Taken together, it seems very likely that PD98059, U0126 and PD184532 act as allosteric inhibitors: they bind outside the ATP- and ERK1/2-binding sites on MEK1/2 and the modification of the three-dimensional structure of MEK1/2 renders it not phosphorylatable by upstream kinases. Such a modification of MEK1/2 may also reduce their kinase activity towards ERK1/2; a high concentration of U0126 and of PD184352 has been shown to inhibit MEK activity directly (41).

Although PD98059, U0126 and PD184352 are highly specific to MEK1/2, they also inhibit the activation of the MEK5-ERK5 pathway at a similar concentration range that inhibits the activation of the MEK1/2-ERK1/2 pathway (44), and inhibition of cyclooxygenase 2 activity by PD98059 has been reported (45).

PD98059 and U0126 completely suppress the proliferation of RPMI-SE (renal cell carcinoma) and HT1060 (fibrosarcoma) cells in which the ERK pathway is constitutively activated, through a mechanism that involves the up-regulation of $p27^{Kip1}$, association of $p27^{Kip1}$ with cyclin E-cyclin-dependent kinase (CDK) 2 complexes, inhibition of cyclin E-CDK2 kinase activity, and a consequent decrease in the phosphorylation state of the retinoblastoma protein. PD98059 also induces a modest apoptotic response in these tumor cells (16). Furthermore, PD184352 exhibits a prominent growth inhibitory effect on human colon tumor xenografts in mice (colon 26 and HT-29 carcinomas) when given orally every 12 h for 2 weeks. Under such conditions, phosphorylation (activation) of ERK1/2 is efficiently suppressed. PD184352 also decreases the invasiveness and motility of HT-29 cells (17). It is currently being evaluated in Phase I clinical trials.

A series of 3-cyano-4-(phenoxyanilino)quinolines has been developed as MEK inhibitors by Wyeth-Ayerst (46). The mechanism of action of these compounds as MEK inhibitors has not been reported. The most potent, Compound 14, inhibits MEK1 activity *in vitro* with an $IC_{50}$ of 2.4 nM and inhibits the proliferation of Colo205, Lovo and SW620 human colon carcinoma cells in culture with $IC_{50}$ of 0.2~0.4 μM.

Several resorcylic acid lactones isolated from microbial extracts display potent inhibitory activity toward MEK. For instance, Ro 09-2210, isolated from a fungal broth FC2506 (47), and L-783,277 ,purified from organic extracts of *Phoma sp.* (ATCC 74403) (48), inhibit MEK1 activity *in vitro* with $IC_{50}$ of 60 nM and 4 nM, respectively. Unlike PD98059, U0126 and PD184352, these compounds are competitive with ATP, and the inhibition is irreversible. These compounds are also effective in cell-based assays, *e.g.*, Ro 09-2210 inhibits phorbol ester-induced activation of AP1 with an $IC_{50}$ below 10 nM, while L-783,277 inhibits the growth of several epithelial tumor cells in soft agar with $IC_{50}$ of 100~200 nM.

**Other inhibitors**

Recently, a peptide corresponding to the amino-terminal 13 amino acids of MEK1 (MPKKKPTPIQLNP), a region intimately involved in the association of ERK1/2 with MEK1, has been shown to specifically inhibit the activation of ERK1/2 (49). The free peptide inhibits ERK activation *in vitro* but not *in vivo* because of its inability to cross cellular membranes. However, inclusion of either an alkyl moiety or a membrane-translocating peptide sequence facilitates the cellular uptake of the peptide inhibitor and prevents ERK activation in phorbol ester-stimulated NIH3T3 cells and NGF-treated PC12 cells with $IC_{50}$ of 13~45 μM.

So far, no specific and potent inhibitors of ERK1/2 have been reported. Recently, purvalanol, one of the most potent cyclin-dependent kinase (CDK) inhibitors to date, has been shown to target ERK1 and ERK2 (50). The anti-proliferative property of purvalanol is mediated by the inhibition of both ERK1/2 and CDKs.

## FUTURE PERSPECTIVES

The ERK pathway represents an attractive therapeutic target, especially for the development of anticancer drugs. Several potent inhibitors of the ERK pathway have been reported, but the development of new small-molecule inhibitors with enhanced bioavailability remains a current priority. Clarification of the three-dimensional structures of Raf-1/B-Raf, MEK1/2 and ERK1/2 will help markedly the development of specific inhibitors against them. Also, detailed structural information on the binding modes of such inhibitors will significantly enhance the drug design process. Given the ubiquitous role of the ERK pathway in the regulation of normal cell functions, it is important to minimize undesirable effects of inhibitors on normal cells functions. In this respect, tumor cells with a constitutively high level of ERK activation appear to be most sensitive to MEK inhibitors (16). Furthermore, oral administration of PD184352 every 12 h for 2 weeks to mice implanted with subcutaneous colon tumors inhibited tumor growth ~80%, but was not accompanied by unacceptable side effects, (17).

Another promising avenue is the therapeutic exploitation of small interfering RNA (siRNA). These double-stranded RNAs of 19 nucleotides in length are extremely potent and specific in targeting the destruction of any given mRNA (51). Indeed it is possible to specifically ablate one isoform of ERK (unpublished results) or even to target specifically a point-mutated form of a *ras* allele without affecting the wild type form. Because siRNAs can work at very low concentration, combining siRNA with delivery using a specific tumor-targeting antibody (52) might represent a new and exciting "genomic surgery" approach.

Inhibitors of the ERK pathway can be classified as "cytostatic" rather than "cytotoxic" anticancer drugs. Such cytostatic agents may selectively inhibit the abnormal activation of their corresponding target molecule and suppress tumor cell growth but without killing the tumor cells. For example, although specific blockade of the ERK pathway by treatment with PD98059 completely suppresses the growth of tumor cells in which the pathway is constitutively activated, it shows only a modest effect on the induction of apoptosis (16). Further, since many of the MEK inhibitors described above are reversible, their removal would permit the re-initiation of tumor cell proliferation; the majority of tumor cells are just "resting" but not dying under the presence of such cytostatic inhibitors. However, a combination of MEK inhibitors and vincristine, for example, markedly enhances the apoptosis-inducing activity of the latter not only in tumor cells *in vitro* but also in human colon tumor xenografts *in vivo* (53).

Combination therapy is particularly important in cancer chemotherapy. It has generally been driven by safety considerations, *i.e.*, combination of cytotoxic agents with non-overlapping toxicities. As discussed above, specific inhibition of the ERK pathway may enhance the anticancer effect of a wide variety of cytotoxic chemotherapeutic agents (27). As a number of ERK pathway inhibitors enter clinical trials, there is considerable optimism that the combination of cytostatic ERK pathway inhibitors and established cytotoxic anticancer drugs will provide a new avenue of treatment.

## ACKNOWLEDGEMENTS

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